Negative Regulation of Ros Receptor Tyrosine Kinase Signaling: An Epithelial Function of the SH2 Domain Protein Tyrosine Phosphatase SHP-1

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Abstract. Male "viable motheaten" (*me^v*) mice, with a naturally occurring mutation in the gene of the SH2 domain protein tyrosine phosphatase SHP-1, are sterile. Known defects in sperm maturation in these mice correlate with an impaired differentiation of the epididymis, which has similarities to the phenotype of mice with a targeted inactivation of the Ros receptor tyrosine kinase. Ros and SHP-1 are coexpressed in epididymal epithelium, and elevated phosphorylation of Ros in the epididymis of *me^v* mice suggests that Ros signaling is under control of SHP-1 in vivo. Phosphorylated Ros strongly and directly associates with SHP-1 in yeast two-hybrid, glutathione *S*-transferase pull-down, and coimmunoprecipitation experiments. Strong binding of SHP-1 to Ros is selective compared to six other receptor tyrosine kinases. The interaction is mediated by the SHP-1 NH_2-terminal SH2 domain and Ros phosphotyrosine 2267. Overexpression of SHP-1 results in Ros dephosphorylation and effectively downregulates Ros-dependent proliferation and transformation. We propose that SHP-1 is an important downstream regulator of Ros signaling.

Key words: protein tyrosine phosphatase • regulation • receptor tyrosine kinase • epididymis • fertility

Introduction

The transmembrane tyrosine kinase Ros, encoded by the protooncogene *c-ros*, is an "orphan" receptor with exclusive expression in specific epithelia (Sonnenberg et al., 1991; Sonnenberg-Riethmacher et al., 1996). The first discovered oncogenic variants of *c-ros* were found to encode proteins with truncated extracellular domain, and they were detected in a chick retrovirus and in human tumor cell lines (Neckameyer and Wang, 1985; Birchmeier et al., 1986). The oncogenic potential of the Ros tyrosine kinase has also been demonstrated by ligand-dependent transformation of NIH3T3 fibroblasts, which were stably transfected with a chimeric receptor consisting of the TrkA/nerve growth factor (NGF)[1] receptor extracellular domain and the Ros transmembrane and cytoplasmic domains (Riethmacher et al., 1994). The physiological function of Ros has been characterized in mice with a targeted mutation of *c-ros*. Male $Ros^{-/-}$ mice exhibit defects in differentiation and regionalization of the epididymal epithelium and, because of this defect, are sterile (Sonnenberg-Riethmacher et al., 1996).

The SH2 domain protein tyrosine phosphatase (PTP) SHP-1 (Shen et al., 1991) is expressed in hematopoietic and, at lower levels, epithelial cells. In the latter cell type, SHP-1 expression is driven by a cell type–specific promoter, which leads to the generation of an epithelial-specific SHP-1 variant (Banville et al., 1995). Multiple binding partners and substrates for SHP-1 have been identified in hematopoietic cells: SHP-1 negatively regulates the signaling of cytokine receptors, receptor tyrosine kinases, adhesion receptors, and immunoreceptors (for reviews see Feng and Pawson, 1994; Frearson and Alexander, 1997; Neel and Tonks, 1997). "Motheaten" (*me*) or "viable motheaten" (*me^v*) mice carry mutations in the SHP-1 gene, which lead to a complete loss or a 80–90% reduction of SHP-1 activity, respectively (Green and Shultz, 1975; Shultz et al., 1984, 1993). Homozygous *me* and *me^v* mice exhibit multiple abnormalities, including immunodeficiencies, increased proliferation of macrophage, neutrophil,

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[1]*Abbreviations used in this paper:* aa, amino acid; ATc, anhydrotetracycline; Erk, extracellular signal regulated kinase; GST, glutathione *S*-transferase; *me*, motheaten; *me^v*, viable motheaten; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PTP, protein tyrosine phosphatase; RTK, receptor tyrosine kinase.

and erythrocyte progenitors (Shultz et al., 1997), and decreased bone density, which is a result of elevated osteoclast activity (Umeda et al., 1999). Cells isolated from *me* and *me^v* mice allowed the identification of SHP-1 target proteins in hematopoietic cells (Klingmüller et al., 1995; Chen et al., 1996). In such cells, phosphorylation of the target proteins is elevated, either constitutively or upon activation of the appropriate signal transduction pathways. Homozygous *me* mice die before they attain puberty. In contrast, homozygous *me^v* mice reach a mean age of 8 to 9 wk, and male homozygous *me^v* mice are sterile. The defects in *me^v*/*me^v* mice leading to sterility are incompletely understood. A reduced testosterone level in these mice is associated with impaired spermatogenesis. Interestingly, testosterone treatment rescues spermatogenesis, but is not sufficient to allow for the production of fully fertile sperm (Shultz et al., 1984). This suggests that late stages of sperm maturation in the epididymis are impaired in *me^v*/*me^v* mice, as in Ros$^{-/-}$ mice. The proximal segment of the epididymis, where Ros and SHP-1 are coexpressed, is aberrantly differentiated in *me^v*/*me^v* mice. Therefore, we speculated that the defects in sperm maturation in Ros$^{-/-}$ and *me^v*/*me^v* mice might be related at the molecular level. If Ros and SHP-1 interact in a common signal transduction pathway, impairment of epididymal function might result from inactivation of either gene. Indeed, our analysis revealed that SHP-1 strongly binds Ros and regulates Ros signaling in a negative manner. In *me^v*/*me^v* mice, Ros is hyperphosphorylated, consistent with an aberrant signaling activity in vivo. We propose that Ros is a target of SHP-1 and that deregulated Ros activity in *me^v*/*me^v* mice contributes to male sterility.

Materials and Methods

Reagents, DNAs, and Mice

NGFβ was purchased from Biomol Feinchemikalien GmbH. Polyclonal anti-phosphotyrosine and monoclonal anti–SHP-1 antibodies were obtained from Transduction Laboratories, polyclonal anti–SHP-1 antibodies and the corresponding blocking peptide from Santa Cruz Biotechnology, Inc., and anti-vinculin monoclonal antibodies from Upstate Biotechnology. Antibodies recognizing activated, phosphorylated extracellular signal regulated kinases (Erks, "phospho-p44/42 MAPK antibody") and against Erk kinase ("pan-Erk antibody") were from Cell Signaling Technology, Inc., and Transduction Laboratories, respectively. The polyclonal anti-Ros antibodies (directed against a COOH-terminal peptide of murine Ros) have been described previously (Riethmacher et al., 1994). Human SHP-1 cDNA was provided by Drs. A. Ullrich and R. Lammers (Max-Planck-Institute für Biochemie, Martinsried, Germany), and cDNAs for murine SHP-1 and human platelet-derived growth factor (PDGF)β receptor were obtained from Dr. M. Thomas (Washington University School of Medicine, St. Louis, MO) and Drs. L. Claesson-Welsh and C.H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden), respectively. The chimerical TrkA-Ros (cloned in the eukaryotic expression vector pEFBOS) has been described previously (Riethmacher et al., 1994; Sachs et al., 1996). SHP-1 mutant *me^v* mice (C57aBL/6J-Hcph *me^v* [stock no. 000811], heterozygous breeding pairs and homozygous males) and control animals (C57BL/6J) were purchased from the Jackson Laboratory. Genotyping was performed by PCR according to a protocol provided by the Jackson Laboratory.

Expression of SHP-1 and Characterization of Epididymal Tissue

Expression analysis of SHP-1 in murine epididymis was carried out using in situ hybridization. Labeled antisense riboprobe (corresponding to nucleotides 164–1,349 in the murine SHP-1 cDNA; Matthews et al., 1992) and a corresponding sense control, were prepared by in vitro transcription in the presence of [^{35}S]CTP and [^{35}S]UTP using T7 and T3 RNA polymerases, respectively. In situ hybridization was carried out as described previously (Sonnenberg et al., 1991). Expression of SHP-1 in human epididymis was analyzed by immunohistochemistry. The anti–SH-PTP1 antibody C-19/sc-2872 (Santa Cruz Biotechnology, Inc.) was incubated with 5-μm tissue cryosections overnight at 4°C. After washing, the sections were treated with a mouse anti–rabbit antibody (1:400; Dako), subsequently with a rabbit anti–mouse immunoglobulin (1:70; Dako), and then with a mouse APAAP (alkaline phosphatase monoclonal anti-alkaline phosphatase) complex (Dako). Naphtol-AS-biphosphate (Sigma-Aldrich) and new fuchsin (Merck) were used as substrate and developer, respectively. To inhibit endogenous tissue enzyme activity, the developing solution was supplemented with 0.25 mM levamisole (Sigma-Aldrich). To evaluate the specificity of immunostaining, the primary antibody was replaced by nonimmune serum, or the incubation was done in the presence of 100 μg/ml of the corresponding blocking peptide.

To monitor tyrosyl phosphorylation in *me^v* mice, the animals were challenged with peroxovanadate (Ruff et al., 1997). For this, a pervanadate solution was prepared by mixing a 5 mM solution of Na_3VO_4 with 30X H_2O_2 to a final H_20_2 concentration of 50 mM, and incubation was for 15 min at room temperature. This solution was injected intraperitoneally (10 μl/g body weight). After 10 min, the mice were killed; the epididymis, intestine, and stomach were prepared and preserved either for histological examination or lysis. For lysis, the epididymis was shock frozen. The tissue was lysed in lysis buffer (~ 1 ml/2 glands) with the aid of a Dounce homogenizer. The lysates were centrifuged at 100,000 *g* at 4°C for 30 min and passed through a 0.22-μm filter. Lysate aliquots (~30 μg of total protein) were used for SDS-PAGE and immunoblot analyses. Equal amounts of lysates (500 μl, protein amounts equilibrated with lysis buffer) were used for anti-Ros immunoprecipitations (10 μl anti-Ros antiserum/reaction).

Paraffin sections of murine epididymis were stained by hematoxylin/eosin.

Expression Constructs

The TrkA-Ros chimera was recloned from pEFBOS to pcDNA3 using EcoRI. Point mutations were introduced into the TrkA-Ros cDNA using an M13 mutagenesis kit (Bio-Rad Laboratories), according to the manufacturer's instructions. Mammalian expression constructs for SHP-1 and various derivatives have been described previously (Tenev et al., 1997; Keilhack et al., 1998), as were the bacterial glutathione *S*-transferase (GST)–fusion expression constructs of SHP-1, SHP-1 SH2 (tandem SH2 domains), SHP-1 R32K, SHP-1 R138K (Tenev et al., 1997), and SHP-1 Δ41 (Frank et al., 1999). The construct for the production of a GST fusion protein of the isolated NH_2-terminal SH2 domain was generated by the digestion of pGEX-5X-1 SH2 (see above) with EcoRI and SpeI. This fragment was cloned into a modified pGEX-5X-1 vector (with an additional XbaI site from pBKS) opened with EcoRI and XbaI. The COOH-terminal SH2 domain was cloned by digestion of pGEX-5X-1 SH2 with SpeI and SmaI. The fragment was treated with Pfu to create blunt ends and cloned into pBKS, which was digested with SmaI. Subsequently the fragment with the proper orientation was excised using EcoRI and NotI from pBKS COOH-SH2 and ligated into pGEX-5X-1, which was opened with EcoRI and NotI. SHP-1 CS was subcloned from pBKS to pGEX-5X-1 using EcoRI and NotI.

Yeast Two-Hybrid Assays

Constructs and the method for determining the interaction strength of a panel of receptor tyrosine kinase cytoplasmic domains with various molecules containing phosphotyrosine interaction domains were described earlier (Weidner et al., 1996; Bai et al., 1998; Tamura et al., 1999; Vayssiere et al., 2000). Interaction strength was assigned qualitatively by visual inspection of yeast growth in the appropriate selection medium compared to other interaction partners as "strong," "weak," or "not detectable" (see Fig. 4 A and Table I). For quantitative β-galactosidase assays, cDNA fragments representing the entire cytoplasmic domain of human PDGFβ receptor (Claesson-Welsh et al., 1988; amino acid [aa] 558–1,106) and murine c-Ros (Riethmacher et al., 1994; aa 1,880–2,339) were cloned into the pLexA vector (CLONTECH Laboratories, Inc.). cDNA sequences corresponding to the tandem SH2 domains and hinge domain of human SHP-1 (aa 1–270) or SHP-2 (aa 1–271) were cloned into pB42AD (CLONTECH Laboratories, Inc.). Expression of all fusion proteins and autophosphorylation activity of kinase constructs were verified by immunoblotting. Expression constructs for the tested interaction partners were cotransformed into *Saccharomyces cereviseae* strain EY48 (CLONTECH Laboratories, Inc.), and individual colonies were spotted multiply on agar plates with "induction medium" (synthetic dropout medium, Gal, Raf, −His, −Trp,

−Ura) containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Growth was allowed for several hours, then the plates were scanned with a flat-bed scanner, and the mean intensity of color development was determined by densitometric evaluation with the program NIH Image 1.57. Signals in the presence of both interaction partners were corrected by subtraction of signals obtained with yeast transfected with expression constructs for the kinase baits and empty pB42AD.

Transient Transfection, Immunoprecipitation, and Dephosphorylation Assay

293 cells were transfected with expression constructs for TrkA-Ros or TrkA-Ros mutants, SHP-1, SHP-1 CS, or empty vector using calcium phosphate coprecipitation (Lammers et al., 1993). For coimmunoprecipitation, 10 μg pRK5RS SHP-1 or SHP-1 C455S DNA was cotransfected with 10 μg pcDNA3 TrkA-Ros DNA per 10-cm dish. For assessment of dephosphorylation, 0.5 μg pcDNA3 TrkA-Ros was cotransfected with 3.5 μg pRK5RS SHP-1, pcDNA3 SHP-1 C455S, or empty vector in 6-well plates. For titration of SHP-1 efficacy, variable amounts of pRK5RS SHP-1 were used (see the legend to Fig. 5 E). After transfection, the cells were stimulated with 50 ng/ml NGFβ or vehicle for 10 min and lysed in 700 (10-cm dish) or 200 μl (6-well plate) lysis buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 20 μM zinc acetate, 50 mM NaF, 10 mM NaPP, 1 mM Na_3VO_4, 1% Triton X-100, 1 mM PMSF, 1 μg/ml pepstatin A, 2 μg/ml aprotinin, 10 μg/ml leupeptin). The lysates were clarified by centrifugation at 25,000 *g* and 4°C for 20 min. For coimmunoprecipitation, 10 μl anti-Ros antiserum or 1 μg monoclonal anti–SHP-1 antibody were used. Immunoprecipitation was carried out as described, and associated proteins were analyzed by SDS-PAGE and immunoblotting. For dephosphorylation assays, lysate aliquots were resolved by SDS-PAGE, and the tyrosyl phosphorylation or activation of endogenous Erk was visualized by immunoblotting.

GST Pull-down Assays and Sequential Immuno and Affinity Precipitations

For pull-down assays using different fusion proteins of SHP-1 domains, 20 μg pcDNA3 TrkA-Ros was transfected into 293 cells (10-cm dishes). The cells were stimulated with 50 ng/ml NGFβ for 10 min and lysed in 700 μl lysis buffer. GST pull-down assays were done, as described previously (Keilhack et al., 1998), using 20 pmol of each fusion protein. Sequential immuno- and affinity precipitations to monitor direct phosphotyrosine-mediated interactions were carried out as described previously (Keilhack et al., 1998). In brief, 20 μg pcDNA3 TrkA-Ros or point mutants were transfected into 293 cells (10-cm dishes). After transfection, the cells were lysed and TrkA-Ros was immunoprecipitated. The immunoprecipitates were denatured and partially renatured by dilution with lysis buffer. These partially renatured lysates were applied to 2 μg GST-SH2 immobilized on glutathione-Sepharose (Amersham Pharmacia Biotech). Bound proteins were visualized by SDS-PAGE and immunoblotting.

Establishment of Inducible SHP-1–expressing NIH3T3 TrkA-Ros Cells

NIH3T3 cells stably transfected with TrkA-Ros (using G418 resistance for selection) (Riethmacher et al., 1994) were supertransfected with SHP-1 DNA in the tetracycline-regulated expression vector pNRTIS-21 (pNRTIS SHP-1) (Tenev et al., 2000) and pSBC (with a hygromycin resistance gene) (Tenev et al., 2000) in a 5:1 ratio in 24-well plates using Superfect (QIAGEN), according to the manufacturer's instructions. The cells were selected with 100 ng/ml anhydrotetracycline (ATc), 200 μg/ml hygromycin B, and 400 μg/ml G418 until colonies formed. The cells were then amplified and clones were generated using limited dilution. Clones that inducibly expressed SHP-1 upon ATc withdrawal were selected for further analysis. Tyrosyl phosphorylation of TrkA-Ros in these cell clones was tested after the cells were grown for 5 d in the absence or presence of 100 ng/ml ATc (six-well plates). Thereafter, cells were treated with 100 or 200 ng/ml NGFβ or vehicle and lysed in 200 μl lysis buffer. TrkA-Ros was immunoprecipitated and its tyrosyl phosphorylation was assessed using SDS-PAGE and immunoblotting. To monitor growth, the cell clones were grown with or without 100 ng/ml ATc for 5 d. Thereafter, 5×10^4 cells were seeded into six-well plates and kept for 8 d with or without 100 ng/ml ATc and in the absence or presence of 50 ng/ml NGFβ. The medium was changed every second day. Subsequently, the cells were trypsinized and counted. The results are represented as fold growth and compared to the seeded number of cells. For focus assays, cell monolayers were grown in the absence or presence of 50 ng/ml NGFβ for 12 d (the medium was changed every second day). Subsequently, the monolayers were stained with crystal violet dye and photographed. Alternatively, a small number of transfected cells were tested for their ability to form foci in a monolayer of wild-type NIH3T3 fibroblasts. 20 transfected cells were mixed with 10^6 wild-type cells and seeded in 6-cm dishes. The cells were kept for 14 d with or without 100 ng/ml ATc in the absence or presence of 50 ng/ml NGFβ (the medium was changed every second day), and foci were counted after staining the plates.

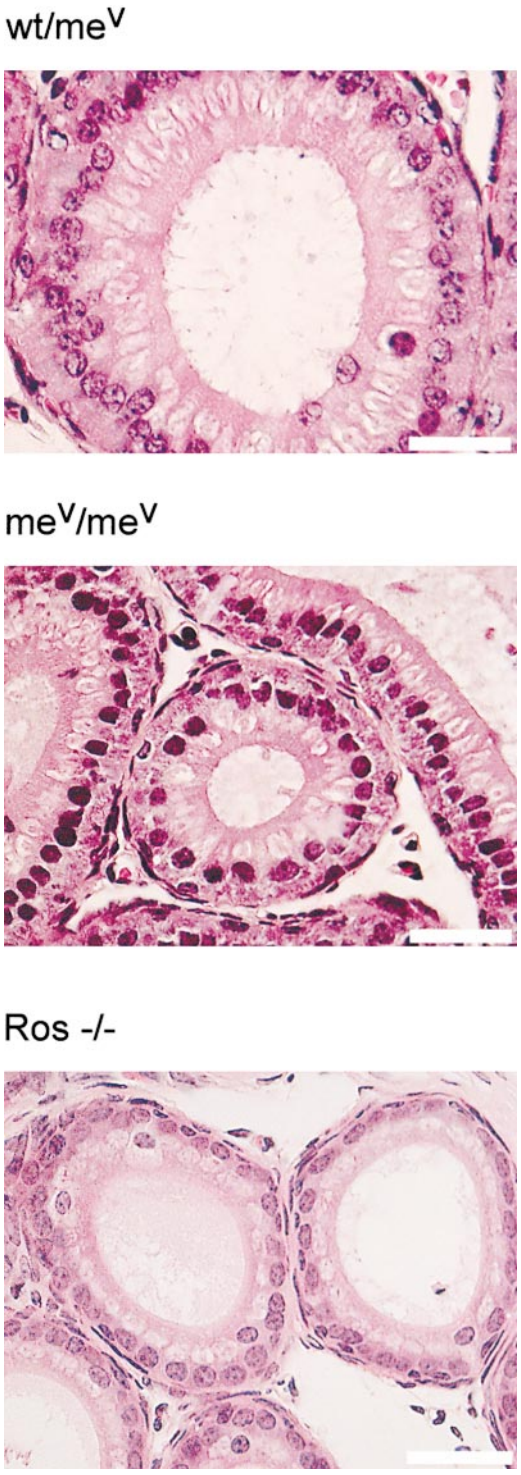

Figure 1. Epididymal differentiation is impaired in homozygous *me^v* mice. Paraffin sections of the epididymis of heterozygous, *me^v/me^v*, and Ros$^{-/-}$ mice were stained with hematoxylin/eosin. Depicted sections represent the proximal segment of the epididymal caput at a magnification of 64×. Bars, 30 μm.

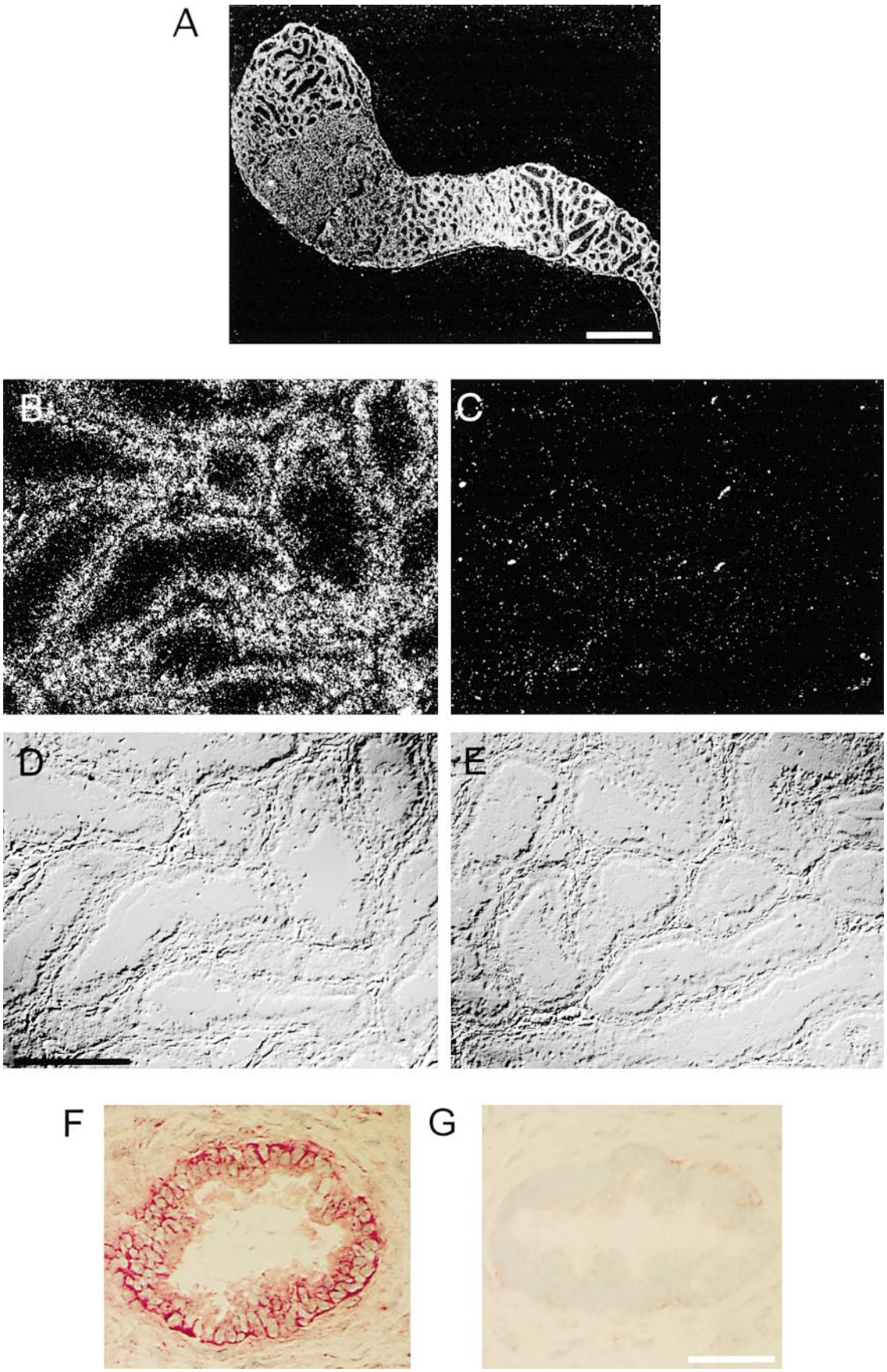

Figure 2. SHP-1 is expressed in the epididymal epithelium. A–E show the expression of SHP-1 mRNA in murine epididymis (wild-type animals) by in situ hybridization. (A) Overview in dark-field representation (antisense riboprobe). Bar, 1 mm. (B) SHP-1 mRNA expression in the proximal tubules at higher magnification. (C) Sense riboprobe control. (D and E) Sections from B and C, respectively, shown in differential interference contrast. Bar, 200 μm. (F) Expression of SHP-1 in human epididymis by immunocytochemistry with anti–SHP-1 antibodies. (G) Negative control for F. The incubation with the primary antibody was performed in the presence of excess antigenic peptide. Bar, 50 μm.

Results

Ros Is Hyperphosphorylated in the Epididymis of me^v Mice

To assess whether SHP-1 might be relevant for epididymal function, we histologically examined the epididymis of male *me^v*/*me^v* mice and compared them to heterozygous controls. Macroscopically, the epididymis of *me^v*/*me^v* mice was smaller, though the overall shape appeared normal. This size reduction was proportional to the reduced body weight of the *me^v*/*me^v* mice. Histological sections revealed that the tall columnar epithelial cells, which form the tubules of the proximal segment in heterozygous animals, are replaced by considerably flatter epithelial cells in the epithelium of *me^v*/*me^v* mice (Fig. 1). In heterozygous animals, these epithelial cells are clearly structured. The nucleus is located above a clearly defined basal cytoplasmic zone. The cytoplasmic zone above the nucleus is broad and the apical surface is rough. In contrast, the nuclei in epithelial cells of *me^v*/*me^v* are often located closer to the basal membrane, and the apical surfaces appear smooth (Fig. 1). The epithelium in the more distal segments of the epididymis exhibited little differences in mutant and control animals (not shown). Histologically, the epithelium of the proximal segment in *me^v*/*me^v* mice has similarities to that of Ros$^{-/-}$ mice (Fig. 1). We also analyzed the epithelium of other organs. Epithelial cells in the intestine of *me^v*/*me^v* mice were smaller than their counterparts in heterozygous animals, but appeared fully differentiated. No differences between *me^v*/*me^v* and heterozygous animals were detectable in epithelia of stomach and pancreas (not shown). In summary, the epididymal epithelium of *me^v*/*me^v* mice exhibits signs of aberrant differentiation. This phenotype may be related to a loss of function of SHP-1 in the epididymal epithelial cells, since SHP-1 is clearly expressed in these cells (Fig. 2). In situ hybridization with an antisense RNA generated from a 1,184-bp fragment of murine SHP-1 cDNA revealed expression of SHP-1 mRNA in the epithelial cells surrounding the tubules of the initial epididymal segment (Fig. 2, A–E). More distal segments exhibit low level signals. Thus, SHP-1 expression mirrors the one of Ros in this part of the epididymis (Sonnenberg-Riethmacher et al., 1996). Strong SHP-1 expression is also seen in the corpus with highest levels in the most distal segments. These distal epithelia are devoid of Ros (Sonnenberg-Riethmacher et al., 1996). SHP-1 expression could also be detected by immunoblotting in the epididymis of mice (not shown); immunostaining failed with the available antibodies. However, in human epididymis, strong epithelial SHP-1 expression could be visualized by immunohistochemistry (Fig. 2, F and G). Thus, SHP-1 is expressed in epithelial cells of the epididymis and exhibits an overlapping expression domain with Ros.

The overlapping expression of SHP-1 and Ros prompted us to test whether impairment of SHP-1 activity in *me^v*/*me^v* mice might affect Ros signaling. We analyzed tyrosine phosphorylation of immunoprecipitated Ros from the epididymis of *me^v*/*me^v* mice. Ros from *me^v*/*me^v* mice displayed an elevated tyrosine phosphorylation compared to Ros from heterozygous animals (Fig. 3), indicating that SHP-1 is capable of downregulating Ros signaling in the epithelium of the epididymis in vivo. Intraperitoneal injection of peroxovanadate rapidly triggers tyrosine phosphorylation in multiple murine tissues (Ruff et al., 1997). After treatment with

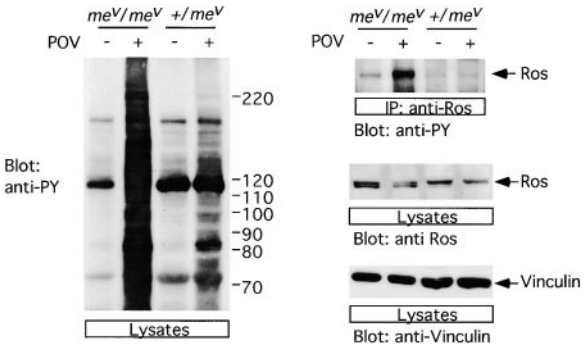

Figure 3. SHP-1 affects Ros signaling in vivo. *me^v*/*me^v* mice or heterozygous control animals were challenged with peroxovanadate (POV) or mock treated (−). The epididymis was prepared and the tissue was lysed. Lysates were used to analyze the phosphotyrosine content (left gel, anti-PY) by immunoblotting. In addition, lysates were used for immunoprecipitation (IP) of Ros and for the subsequent analysis of Ros phosphorylation, by immunoblotting (right gel). The data are representative of three independent experiments with consistent results.

peroxovanadate, a dramatic increase in overall tyrosyl phosphorylation was detected in epididymal extracts from *me^v*/*me^v* mice, which was less pronounced in heterozygous mice (Fig. 3). In particular, phosphorylation of Ros was strongly elevated in peroxovanadate-treated homozygous, but not in heterozygous *me^v* mice. Peroxovanadate is an effective inhibitor of many PTPs, but a relatively weak inhibitor of SHP-1 (Wetzker, M., and F.D. Böhmer, unpublished data). We assume that partial inhibition of SHP-1 by the pervanadate treatment eliminates the residual activity of SHP-1 in the *me^v*/*me^v* mice, but leaves substantial activity in the heterozygous mice intact, resulting in a further enhancement of the difference in Ros phosphorylation level.

Ros and SHP-1 Exhibit a Strong and Direct Interaction

To further investigate the regulation of Ros by SHP-1, we tested whether SHP-1 is a direct binding partner for Ros in a yeast two-hybrid assay. The cytoplasmic domain of Ros was fused to the DNA-binding domain of LexA. In such constructs, the tyrosine kinase of the fusion proteins is constitutively active, phosphorylated, and can interact with cognate binding partners (Weidner et al., 1996). We observed very strong binding between the SH2 domains of SHP-1 and the cytoplasmic domain of Ros (Table I).

Table I. Interaction of Various Autophosphorylated Receptor Tyrosine Kinases with SHP-1 and Other Signaling Proteins in Yeast Two-Hybrid Assays

RTK (cytoplasmic domain fusion protein as bait)	Binding strength of SHP-1 SH2 domains*	Strongly binding signaling molecules
Ros	Strong	PLCγ, c-Abl SH2
TrkA§	Not detectable	PLCγ
Insulin receptor	Not detectable	p85α
IGF-1 receptor§	Not detectable	p85α
CSF-1 receptor§	Not detectable	Grb2, Grb10
Met/HGF receptor§	Weak	Gab1, Grb2

*The interactions were tested in yeast colony growth assays as shown in Fig. 4 A. See text for further description of the signaling proteins.
§TrkA, NGF receptor; IGF-1, insulin-like growth factor-1; CSF-1, colony-stimulating factor-1; HGF, hepatocyte growth factor.

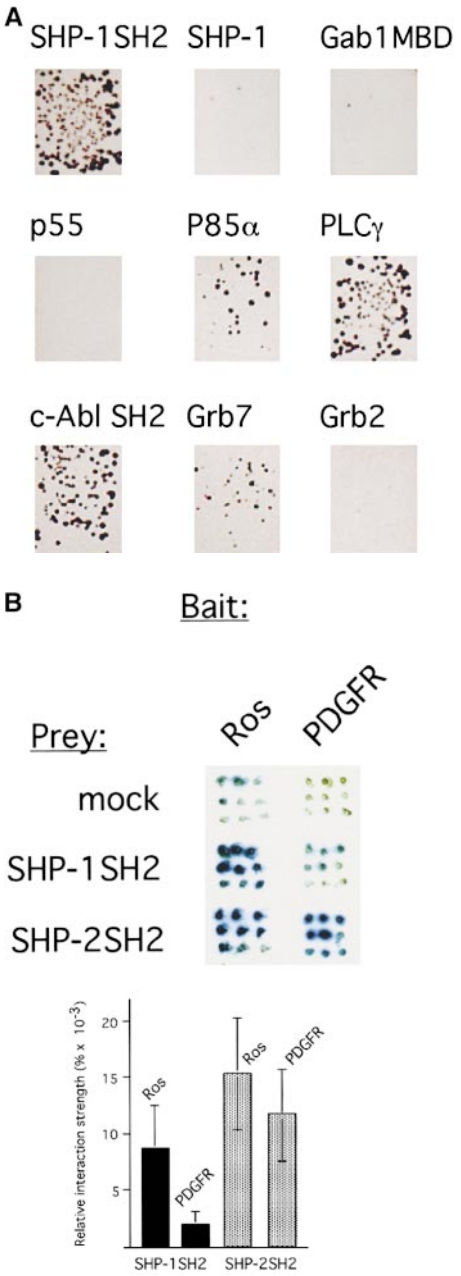

Figure 4. The SHP-1 SH2 domains are strong binding partners for Ros in yeast two-hybrid assays. (A) The interaction of various signaling molecules as "prey" (indicated) with the autophosphorylated cytoplasmic domain of Ros as "bait" was tested in yeast colony growth assays. (B) The interaction of the SH2 domains of either SHP-1 or the related PTP SHP-2 with Ros or, for comparison, with the PDGFβ receptor was quantitatively measured with a β-galactosidase reporter gene assay. Colonies were spotted on X-gal–containing plates and color development, as depicted in the top picture, were quantitated by densitometry (graph, mean ± SD, $n = 6$).

Other tested receptor tyrosine kinases (RTKs) exhibited little or no detectable interaction with SHP-1 SH2 domains, but interacted strongly with other partners. Thus, the direct binding of SHP-1 to Ros is remarkably specific compared to other tested receptor baits. In addition to the SH2 domains of SHP-1, phospholipase Cγ, the SH2 domain of c-Abl, the phosphoinositide-3 kinase subunit p85α, Grb10, and the SH2 domains of SHP-2 were strong binding partners for Ros, whereas the signaling adapter proteins Shc, Grb7, Crk, and Nck bound to a lesser extent. The adapter protein Grb2, the Gab1 Met-binding domain, GTPase-activating protein, and the phosphoinositide-3 kinase subunit p55 exhibited no detectable binding (Fig. 4 A, and data not shown). Interestingly, full-length SHP-1 did not interact with Ros in this assay, suggesting that the presence of the catalytic domain of the PTP prevents the formation of a stable complex. Strong interaction of SHP-1 SH2 domains with Ros was confirmed using an alternative yeast two-hybrid assay that allows quantification (Fig. 4 B). Only a weak interaction of the SHP-1 SH2 domains was detectable with the PDGFβ-receptor cytoplasmic domain. For comparison, the SH2 domains of SHP-2 were tested, since binding of SHP-2 to the PDGFβ receptor has been established (Valius et al., 1993). They bound with comparable strength to PDGFβ receptor and Ros baits.

We further tested the interaction between SHP-1 and Ros in mammalian cells, using transient transfections of HEK293 cells. Since the ligand for Ros is unknown, we used a chimerical receptor composed of the extracellular part of human TrkA and the intracellular domain of murine Ros (TrkA-Ros), which is activated in response to NGFβ (Riethmacher et al., 1994). The chimerical receptor was expressed, alone or together, with wild-type SHP-1 or with a catalytically inactive SHP-1 mutant (C455S). Immunoprecipitations demonstrate the formation of a complex between active SHP-1 and TrkA-Ros in NGFβ-stimulated, but not -unstimulated, cells (Fig. 5 A). Moreover, a very strong complex formation occurred between the C455S mutant of SHP-1 and TrkA-Ros, even without ligand addition, which was further enhanced by NGFβ stimulation. Thus, SHP-1 binds Ros also in mammalian cells; catalytic activity of SHP-1 reduces binding, as observed in the yeast two-hybrid system. In addition, "substrate trapping" of SHP-1 C455S may contribute to the very efficient association with Ros. The strong binding of SHP-1 C455S in the absence of NGFβ is likely due to significant constitutive activity of TrkA-Ros tyrosine kinase caused by overexpression.

SHP-1 Binds to Ros Phosphotyrosine 2267 via the NH_2-terminal SH2 Domain

To identify the domains of SHP-1 that are necessary for the interaction with TrkA-Ros, we used GST pull-down assays with HEK293 lysates containing autophosphorylated TrkA-Ros (Fig. 5 B). The lysates were incubated with equal molar amounts of GST, GST fusion proteins that contain full-length SHP-1, and various SHP-1 derivatives. The amount of TrkA-Ros recovered on GSH beads was analyzed by immunoblotting (Fig. 5 B). In such an assay, TrkA-Ros can be precipitated with fusion proteins that contain an intact NH_2-terminal SH2 domain of SHP-1. Very little if any binding was observed to the other SHP-1 subdomains, for instance the isolated COOH-terminal SH2 domain. Thus, the NH_2-terminal SH2 domain plays a dominant role in the interaction with Ros. Guided by the consensus sequence for ligands of the NH_2-terminal SH2 domains of SHP-1, hXY(P)XXh (X = any amino acid and h = hydrophobic

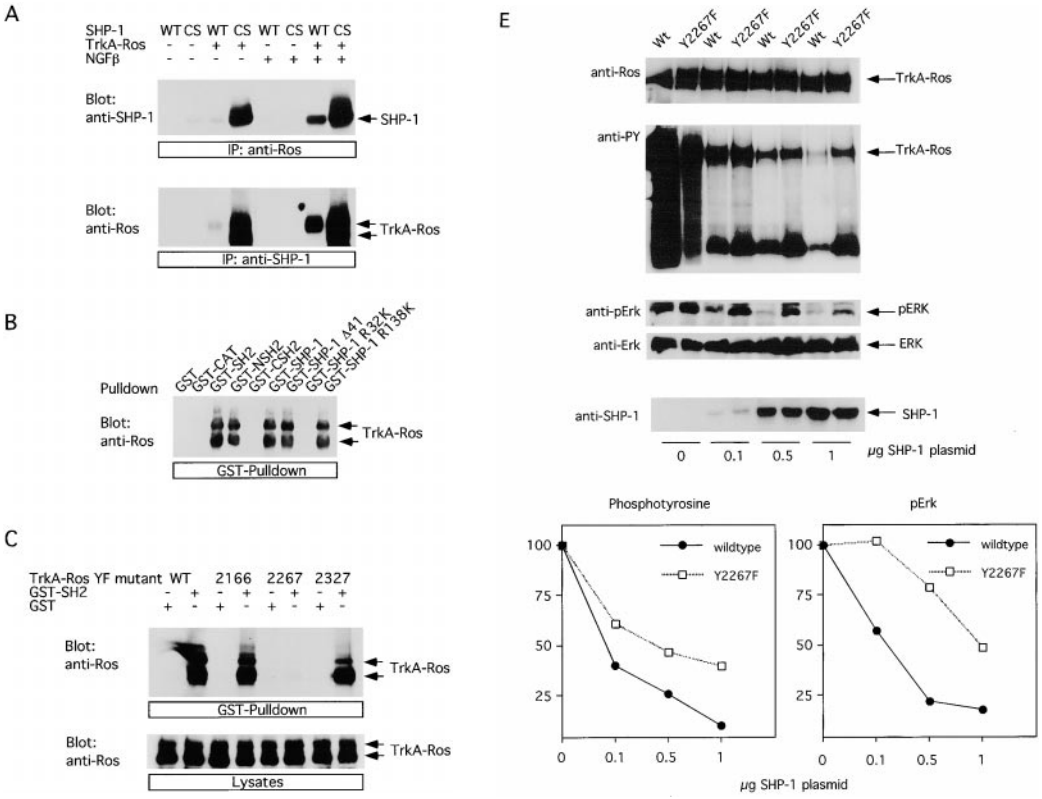

Figure 5. SHP-1 interacts with and dephosphorylates autophosphorylated TrkA-Ros. (A) SHP-1 wild-type (WT) or a catalytically inactive SHP-1 mutant (CS) were coexpressed with TrkA-Ros in HEK293 cells, and the cells were stimulated with NGFβ or vehicle. Cell lysates were used for reciprocal coimmunoprecipitation experiments. (B) HEK293 cell lysates containing autophosphorylated TrkA-Ros were used in GST pull-down assays using different fragments of SHP-1 as GST fusion proteins. CAT, catalytic domain; SH2, SH2 domains; NSH2, isolated NH_2-terminal SH2 domain; CSH2, isolated COOH-terminal SH2 domain; SHP-1, full-length enzyme; SHP-1 Δ41, 41 amino acids deleted at the COOH-terminus; SHP-1 R32K, inactivated NH_2-terminal SH2 domain; SHP-1 R138K, inactivated COOH-terminal SH2 domain. (C) Denatured and partially renatured anti-Ros immunoprecipitates containing wild-type TrkA-Ros or point mutants were used in GST pull-down assays using the isolated SH2 domains of SHP-1 as a GST fusion protein or GST alone (sequential immuno- and affinity precipitation). Expression controls for the different TrkA-Ros variants are shown in the bottom gel. (D) SHP-1 WT and SHP-1 C455S were coexpressed with TrkA-Ros in HEK293 cells, and the cells were stimulated with NGFβ. Lysate aliquots were used to analyze the phosphotyrosine content by SDS-PAGE and immunoblotting. (E) Effect of different amounts of SHP-1 on Ros-dependent tyrosine phosphorylation and Erk activation. TrkA-Ros (wild-type) and TrkA-Ros Y2267F were coexpressed with different amounts of SHP-1 (expression plasmid amounts indicated). Total cell lysates were evaluated for tyrosine phosphorylation and activity of endogenous Erk by immunoblotting with anti-phosphotyrosine (anti-PY) and anti–phospho-Erk (pErk) antibodies. Quantification of Ros tyrosine phosphorylation and phospho-Erk signals is depicted in the graph. Consistent results were obtained in three independent experiments.

amino acids) (Burshtyn et al., 1997), we identified various putative tyrosine residues as potential SHP-1–binding sites. These tyrosine residues in TrkA-Ros were mutated to phenylalanine and the obtained TrkA-Ros mutants were compared to wild-type TrkA-Ros for their ability to interact directly with the SH2 domains of SHP-1. For this, a previously described sequential immuno- and affinity precipitation assay (Keilhack et al., 1998) was employed, which permits only detection of direct interactions, but not binding mediated via adapter proteins. The mutation Y2267F interfered with the interaction of the SHP-1 SH2 domain; the Y2166F and Y2327F mutations had little effect (Fig. 5 C). Thus, phosphotyrosine 2267 in the Ros sequence is the major direct binding site for the SH2 domains of SHP-1.

SHP-1 Dephosphorylates Ros and Inhibits Ros-dependent Cell Growth and Transformation

To assess whether the Ros-SHP-1 interaction results in SHP-1–mediated Ros dephosphorylation, we coexpressed TrkA-Ros with SHP-1 or with the catalytically inactive SHP-1 C455S mutant in HEK293 cells. The cells were stimulated with NGFβ, and the phosphotyrosine content was analyzed by immunoblotting (Fig. 5 D). TrkA-Ros overexpression led to a massive tyrosine phosphorylation

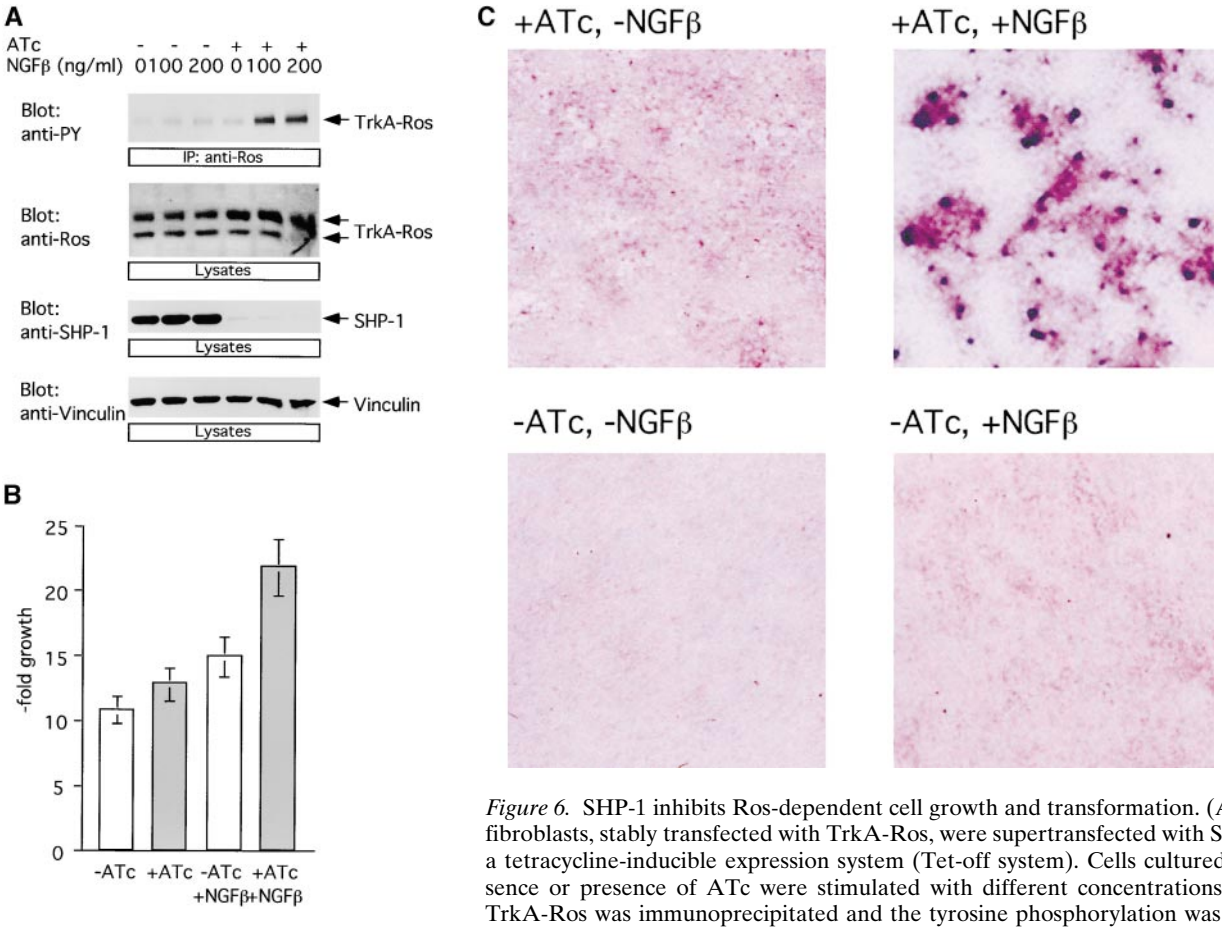

Figure 6. SHP-1 inhibits Ros-dependent cell growth and transformation. (A) NIH3T3 fibroblasts, stably transfected with TrkA-Ros, were supertransfected with SHP-1 using a tetracycline-inducible expression system (Tet-off system). Cells cultured in the absence or presence of ATc were stimulated with different concentrations of NGFβ. TrkA-Ros was immunoprecipitated and the tyrosine phosphorylation was monitored by immunoblotting. (B) The growth of the cells in the absence or presence of ATc and in the absence or presence of NGFβ was analyzed. The results are represented as fold growth after 8 d and compared to the seeded number of cells (mean of triplicates). (C) To analyze focus formation, cell monolayers were grown in the absence or presence of ATc and in the absence or presence of NGFβ for 12 d. Then, the monolayers were stained with crystal violet dye. In an alternative type of assay, we tested focus formation in the presence of an excess of parental NIH3T3 cells. When 20 transfected cells were mixed with 10^6 parental cells, the following numbers of foci per dish were observed in a typical experiment: +ATc, +NGFβ: 9; +ATc, −NGFβ: 2; −ATc, −NGFβ: 0; and −ATc, + NGFβ: 0.

of TrkA-Ros and other proteins. The C455S mutant of SHP-1 had virtually no effect, whereas SHP-1 expression resulted in a complete disappearance of the phosphorylated proteins. We reproducibly observed some reduction of the TrkA-Ros protein level upon SHP-1 transfection; this decrease in expression may contribute, but is by far not sufficient to explain the reduced phosphorylation level of TrkA-Ros. Thus, a very pronounced suppression of Ros-dependent tyrosine phosphorylation is mediated by the catalytic activity of SHP-1. This effect could be accounted for by a direct dephosphorylation of TrkA-Ros and, subsequently, a decrease of its kinase activity and substrate binding and/or by the dephosphorylation of TrkA-Ros substrates.

We compared the relative susceptibility of TrkA-Ros signaling activity with that of the TrkA-Ros Y2267F mutant. The Y2267F mutant exhibited a reduced autophosphorylation and phosphorylation of cellular substrates, presumably resulting from loss of a major autophosphorylation site (Fig. 5 E). However, the level of activation of Erk was similar in cells expressing either TrkA-Ros variant. Consistent with the loss of direct SHP-1 binding, phosphorylation activity of the TrkA-Ros Y2267F mutant and TrkA-Ros Y2267F-dependent Erk phosphorylation were less effectively downregulated by SHP-1 than in the case of wild-type TrkA-Ros. This finding supports the functional importance of direct SHP-1 binding to Ros for negative regulation of signaling activity. Still, signaling of TrkA-Ros Y2267F is negatively affected by SHP-1, in particular, at higher SHP-1 levels (Fig. 5 E), suggesting the additional operation of an indirect Ros-SHP-1 interaction in intact cells.

Proliferation of stably TrkA-Ros–expressing NIH3T3 fibroblasts can be stimulated with NGFβ; upon prolonged NGFβ stimulation, the cells assume a transformed phenotype (Riethmacher et al., 1994). We established derivatives of TrkA-Ros–expressing NIH3T3 cells, which express SHP-1 in a tetracycline-regulated manner. Cell clones containing the inducible SHP-1 expression vector were isolated and tested for the effect of SHP-1 on the TrkA-Ros phosphorylation. To induce or suppress SHP-1 expression, cells were cultured in the absence or presence of ATc. After stimulation with different NGFβ concentrations (Fig. 6 A), phosphorylation of TrkA-Ros was monitored. SHP-1 expression led to a reduction of the NGFβ-stimulated tyrosine phosphorylation of TrkA-Ros. Thus, as in HEK293 cells, TrkA-Ros is an efficient substrate for SHP-1 in NIH3T3 cells.

We further assessed whether the SHP-1–mediated decrease of TrkA-Ros phosphorylation changes the growth behavior of the NIH3T3 fibroblasts. SHP-1 expression was induced (−ATc) or suppressed (+ATc) in cells grown in the absence or presence of NGFβ in low serum. NGFβ treatment resulted in a stimulation of cell proliferation (Fig. 6 B). Expression of SHP-1 attenuated growth of the cells in the absence of NGFβ, and strongly compromised the growth stimulation by NGFβ. Thus, SHP-1 negatively regulates the TrkA-Ros–mediated growth response. When cultured with NGFβ and in the presence of ATc (suppression of SHP-1 expression), the cells spontaneously formed multiple foci (Fig. 6 C). Induction of SHP-1 expression in NGFβ-treated cells completely suppressed focus formation. No foci were formed in the absence of NGFβ, regardless of whether the cells did or did not express SHP-1. Likewise, the ability of a small number of transfected cells to form foci in a monolayer of untransfected NIH3T3 fibroblasts (Riethmacher et al., 1994) was suppressed by SHP-1 expression, as explained in the legend to Fig. 6. In summary, SHP-1 can suppress Ros-dependent phosphorylation, cell proliferation, and transformation.

Discussion

The signal transduction of receptor tyrosine kinases is modulated by PTPs, however, the identity of relevant PTP molecules is often not known. For the SH2 domain PTP SHP-1, a negative regulator of signal transduction, various targets in hematopoietic cells have been identified, including the RTK Kit/SCF receptor (Lorenz et al., 1996; Paulson et al., 1996) and the CSF-1 receptor (Chen et al., 1996). Here, we present evidence for an epithelial target of SHP-1: the "orphan" RTK Ros. We show that SHP-1 and Ros are coexpressed in the proximal segment of the epididymal caput. In agreement with a functional interaction of Ros and SHP-1 in these cells, we observed hyperphosphorylation of Ros in *me^v* mice, whose SHP-1 activity is strongly compromised. Experiments in different cellular models further support that SHP-1 is an effective negative regulator of Ros phosphorylation and signaling.

The effects of SHP-1 appear to be mediated by a remarkably efficient and direct binding to the phosphorylated Ros receptor. Direct binding is mediated by the NH_2-terminal SH2 domain of SHP-1 and phosphotyrosine 2267 in the COOH-terminal part of Ros. The Ros sequence $LNY_{2267}MVL$ matches the known consensus for binding the SHP-1 NH_2-terminal SH2 domain and may mediate a particularly high affinity interaction. Alternatively, a very high stoichiometry of phosphorylation of this site may explain the high efficiency of interaction. Phosphorylated Ros is not only a strong binding partner for SHP-1, but also a very good substrate. Dephosphorylation of Ros appears to include the tyrosine that serves as a binding site of the SHP-1 SH2 domain, which abolishes binding. This would explain why no interaction is observed between Ros and a catalytically active SHP-1 protein in yeast two-hybrid interaction experiments, though Ros and the SH2 domain of SHP-1 interact strongly. Similarly, the amounts of detectable SHP-1–TrkA-Ros complex are highly elevated when a catalytically inactive variant of SHP-1 is used in coimmunoprecipitation and pull-down experiments. Destruction of a SHP-1–binding site by SHP-1–mediated dephosphorylation has been previously observed for the interleukin 3 receptor (Bone et al., 1997). Coexpression of SHP-1 with the TrkA-Ros chimera leads not only to dephosphorylation of TrkA-Ros, but also abolishes tyrosine phosphorylation of other substrates. This could be correlated to dephosphorylation of Ros, which will lead to a decrease of the catalytic activity of its kinase and a decrease of the capacity to recruit substrate proteins. Alternatively, or in addition, Ros substrates may also become directly dephosphorylated by SHP-1.

The hitherto best understood function of Ros is its role in differentiation and regionalization of the epididymal epithelium of mice; the correct development of this epithelium is essential for male fertility. Deregulation of epididymal differentiation in $Ros^{-/-}$ mice leads to sterility (Sonnenberg-Riethmacher et al., 1996). Sterility is due to subtle defects in sperm maturation, which have not yet been characterized at the molecular level (Cooper, 1998; Yeung et al., 1998). It is possible that the phenotypic abnormalities that we have observed in the epididymis of male *me^v/me^v* mice contribute to the sterility of these mice in a similar manner. We observed histological aberrations only in the most proximal segment of the epididymis, i.e., in the region where SHP-1 and Ros are coexpressed. Our results for the SHP-1/Ros interaction in vitro and the observation that Ros is hyperphosphorylated in *me^v/me^v* mice in vivo strongly suggest an elevated level of Ros signaling activity in these animals. It is possible that the histological changes in the caput epididymis of *me^v/me^v* mice are the consequence of deregulated Ros activity. It is obvious that the molecular consequences of Ros inactivation and elevated Ros signaling in epididymal cells will be very different. Interestingly, the phenotypic abnormalities seen in the epididymis of $Ros^{-/-}$ mice and *me^v/me^v* mice have similarities on the morphological level. A more detailed characterization of the functional defects in epididymal differentiation in *me^v/me^v*, as well as $Ros^{-/-}$, mice will be required to better define similarities and differences of the two phenotypes.

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References

Bai, R.Y., T. Jahn, S. Schrem, G. Munzert, K.M. Weidner, J.Y. Wang, and J. Duyster. 1998. The SH2-containing adapter protein GRB10 interacts with BCR-ABL. *Oncogene*. 17:941–948.

Banville, D., R. Stocco, and S.H. Shen. 1995. Human protein tyrosine phosphatase 1C (ptpn6) gene structure—alternate promoter usage and exon skipping generate multiple transcripts. *Genomics*. 27:165–173.

Birchmeier, C., D. Birnbaum, G. Waitches, O. Fasano, and M. Wigler. 1986. Characterization of an activated human ros gene. *Mol. Cell. Biol.* 6:3109–3116.

Bone, H., U. Dechert, F. Jirik, J.W. Schrader, and M.J. Welham. 1997. SHP1 and SHP2 protein-tyrosine phosphatases associate with betac after interleukin-3-induced receptor tyrosine phosphorylation. Identification of potential binding sites and substrates. *J. Biol. Chem.* 272:14470–14476.

Burshtyn, D.N., W. Yang, T. Yi, and E.O. Long. 1997. A novel phosphotyrosine motif with a critical amino acid at position −2 for the SH2 domain-mediated activation of the tyrosine phosphatase SHP-1. *J. Biol. Chem*. 272:13066–13072.

Chen, H.E., S. Chang, T. Trub, and B.G. Neel. 1996. Regulation of colony-stimulating factor-1 receptor signaling by the SH2 domain-containing tyrosine phosphatase SHPTP1. *Mol. Cell. Biol.* 16:3685–3697.

Claesson-Welsh, L., A. Eriksson, A. Moren, L. Severinsson, B. Ek, A. Östman, C. Betsholtz, and C.H. Heldin. 1988. cDNA cloning and expression of a human platelet-derived growth factor (PDGF) receptor specific for B-chain-containing PDGF molecules. *Mol. Cell. Biol.* 8:3476–3486.

Cooper, T.G. 1998. Interactions between epididymal secretions and spermatozoa. *J. Reprod. Fertil. Suppl.* 53:119–136.

Feng, G.S., and T. Pawson. 1994. Phosphotyrosine phosphatases with SH2 domains—regulators of signal transduction. *Trends Genet.* 10:54–58.

Frank, C., H. Keilhack, F. Opitz, O. Zschörnig, and F.D. Böhmer. 1999. Binding of phosphatidic acid to the protein-tyrosine phosphatase SHP-1 as a basis for activity modulation. *Biochemistry*. 38:11993–12002.

Frearson, J.A., and D.R. Alexander. 1997. The role of phosphotyrosine phosphatases in hematopoietic-cell signal-transduction. *Bioessays*. 19:417–427.

Green, M.C., and L.D. Shultz. 1975. Motheaten, an immunodeficient mutant of the mouse. I. Genetics and pathology. *J. Hered*. 66:250–258.

Keilhack, H., T. Tenev, E. Nyakatura, J. Godovac-Zimmermann, L. Nielsen, K. Seedorf, and F.D. Böhmer. 1998. Phosphotyrosine 1173 mediates binding of the protein-tyrosine phosphatase SHP-1 to the epidermal growth factor receptor and attenuation of receptor signaling. *J. Biol. Chem*. 273:24839–24846.

Klingmüller, U., U. Lorenz, L.C. Cantley, B.G. Neel, and H.F. Lodish. 1995. Specific recruitment of SH-PTP1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals. *Cell*. 80:729–738.

Lammers, R., B. Bossenmaier, D.E. Cool, N.K. Tonks, J. Schlessinger, E.H. Fischer, and A. Ullrich. 1993. Differential activities of protein tyrosine phosphatases in intact cells. *J. Biol. Chem*. 268:22456–22462.

Lorenz, U., A.D. Bergemann, H.N. Steinberg, J.G. Flanagan, X. Li, S.J. Galli, and B.G. Neel. 1996. Genetic analysis reveals cell type-specific regulation of receptor tyrosine kinase c-Kit by the protein tyrosine phosphatase SHP1. *J. Exp. Med*. 184:1111–1126.

Matthews, R.J., D.B. Bowne, E. Flores, and M.L. Thomas. 1992. Characterization of hematopoietic intracellular protein tyrosine phosphatases: description of a phosphatase containing an SH2 domain and another enriched in proline-, glutamic acid-, serine-, and threonine-rich sequences. *Mol. Cell. Biol*. 12:2396–2405.

Neckameyer, W.S., and L.H. Wang. 1985. Nucleotide sequence of avian sarcoma virus UR2 and comparison of its transforming gene with other members of the tyrosine protein kinase oncogene family. *J. Virol*. 53:879–884.

Neel, B.G., and N.K. Tonks. 1997. Protein tyrosine phosphatases in signal transduction. *Curr. Opin. Cell Biol*. 9:193–204.

Paulson, R.F., S. Vesely, K.A. Siminovitch, and A. Bernstein. 1996. Signalling by the W/Kit receptor tyrosine kinase is negatively regulated in vivo by the protein tyrosine phosphatase SHP1. *Nat. Genet*. 13:309–315.

Riethmacher, D., O. Langholz, S. Gödecke, M. Sachs, and C. Birchmeier. 1994. Biochemical and functional characterization of the murine ros protooncogene. *Oncogene*. 9:3617–3626.

Ruff, S.J., K. Chen, and S. Cohen. 1997. Peroxovanadate induces tyrosine phosphorylation of multiple signaling proteins in mouse liver and kidney. *J. Biol. Chem*. 272:1263–1267.

Sachs, M., K.M. Weidner, V. Brinkmann, I. Walther, A. Obermeier, A. Ullrich, and W. Birchmeier. 1996. Motogenic and morphogenic activity of epithelial receptor tyrosine kinases. *J. Cell. Biol*. 133:1095–1107.

Shen, S.H., L. Bastien, B.I. Posner, and P. Chretien. 1991. A protein-tyrosine phosphatase with sequence similarity to the SH2 domain of the protein-tyrosine kinases. *Nature*. 352:736–739.

Shultz, L.D., D.R. Coman, C.L. Bailey, W.G. Beamer, and C.L. Sidman. 1984. "Viable motheaten," a new allele at the motheaten locus. I. Pathology. *Am. J. Pathol*. 116:179–192.

Shultz, L.D., P.A. Schweitzer, T.V. Rajan, T. Yi, J.N. Ihle, R.J. Matthews, M.L. Thomas, and D.R. Beier. 1993. Mutations at the murine motheaten locus are within the hematopoietic cell protein-tyrosine phosphatase (Hcph) gene. *Cell*. 73:1445–1454.

Shultz, L.D., T.V. Rajan, and D.L. Greiner. 1997. Severe defects in immunity and hematopoiesis caused by SHP-1 protein-tyrosine-phosphatase deficiency. *Trends Biotechnol*. 15:302–307.

Sonnenberg, E., A. Godecke, B. Walter, F. Bladt, and C. Birchmeier. 1991. Transient and locally restricted expression of the ros1 protooncogene during mouse development. *EMBO (Eur. Mol. Biol. Organ.) J*. 10:3693–3702.

Sonnenberg-Riethmacher, E., B. Walter, D. Riethmacher, S. Gödecke, and C. Birchmeier. 1996. The c-ros tyrosine kinase receptor controls regionalization and differentiation of epithelial cells in the epididymis. *Genes Dev*. 10:1184–1193.

Tamura, T., A. Mancini, H. Joos, A. Koch, C. Hakim, J. Dumanski, K.M. Weidner, and H. Niemann. 1999. FMIP, a novel Fms-interacting protein, affects granulocyte/macrophage differentiation. *Oncogene*. 18:6488–6495.

Tenev, T., H. Keilhack, S. Tomic, B. Stoyanov, M. Stein-Erlach, R. Lammers, A.V. Krivtsov, A. Ullrich, and F.D. Böhmer. 1997. Both SH2 domains are involved in interaction of SHP-1 with the epidermal growth-factor receptor but cannot confer receptor-directed activity to SHP-1/SHP-2 chimera. *J. Biol. Chem*. 272:5966–5973.

Tenev, T., S.A. Böhmer, R. Kaufmann, S. Frese, T. Bittorf, T. Beckers, and F.D. Böhmer. 2000. Perinuclear localization of the protein-tyrosine phosphatase SHP-1 and inhibition of epidermal growth factor-stimulated STAT1/3 activation in A431 cells. *Eur. J. Cell Biol.* 79:1–11.

Umeda, S., W.G. Beamer, K. Takagi, M. Naito, S. Hayashi, H. Yonemitsu, T.L. Yi, and L.D. Shultz. 1999. Deficiency of SHP-1 protein-tyrosine phosphatase activity results in heightened osteoclast function and decreased bone density. *Am. J. Pathol*. 155:223–233.

Valius, M., C. Bazenet, and A. Kazlauskas. 1993. Tyrosine-1021 and tyrosine-1009 are phosphorylation sites in the carboxy terminus of the platelet-derived growth factor receptor-beta subunit and are required for binding of phospholipase C gamma and a 64-kilodalton protein, respectively. *Mol. Cell. Biol*. 13:133–143.

Vayssiere, B., G. Zalcman, Y. Mahe, G. Mirey, T. Ligensa, K.M. Weidner, P. Chardin, and J. Camonis. 2000. Interaction of the Grb7 adapter protein with Rnd1, a new member of the Rho family. *FEBS Lett*. 467:91–96.

Weidner, K.M., S. Di Cesare, M. Sachs, V. Brinkmann, J. Behrens, and W. Birchmeier. 1996. Interaction between Gab1 and the c-Met receptor tyrosine kinase is responsible for epithelial morphogenesis. *Nature*. 384:173–176.

Yeung, C.H., E. Sonnenberg-Riethmacher, and T.G. Cooper. 1998. Receptor tyrosine kinase c-ros knockout mice as a model for the study of epididymal regulation of sperm function. *J. Reprod. Fertil. Suppl*. 53:137–147.